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14. ABSTRACT
Acute alcohol intoxication (AAI) impairs the hemodynamic counteregulatory response to trauma and hemorrhagic shock (HS), blunts the pressor response to fluid resuscitation (FR), suppresses the HS-induced neuroendocrine response, impairs pro-inflammatory cytokine expression and increases mortality from infection during recovery. Studies conducted during this funding period examined a) whether the attenuated neuroendocrine response, particularly reduced sympathetic nervous system (SNS) activation, is the principal mechanism responsible for the hemodynamic instability seen in AAI+ HS and b) what the impact of AAI was on the integrity of host defense mechanisms during the immediate and delayed recovery from HS. We determined whether SNS activation can be restored by central (intracerebroventricular; ICV) neostigmine administration and whether this in turn is capable of improving the hemodynamic counteregulatory response to HS in AAI. Our results show that ICV neostigmine stimulates SNS activation and improves the recovery of blood pressure following hemorrhagic shock. Furthermore, our results indicate that this in part mediated by arginine vasopressin. Interestingly while the presser response to phenylephrine in vitro appears to be blunted by alcohol, the in vivo response to a presser with a different mechanism of action appears to be preserved.

15. SUBJECT TERMS
Alcohol intoxication, hemorrhage, injury, blood pressure, immune function

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INTRODUCTION:

According to the National Trauma Institute, traumatic injury accounts for approximately 37 million emergency department visits and produces an economic burden of over 400 billion dollars in both health care costs and loss of productivity per year (National Trauma Institute Source: CDC). Traumatic injury ranks as the number one cause of death for the 1-44 year old age group and 5th leading cause of death overall (*www.**nationaltraumainstitute**.org/home/**trauma**_statistics.html*). Acute alcohol intoxication (AAI) contributes to the increased risk of traumatic injury; with intoxicating blood alcohol levels present in more than 40% of injured patients (Cherpitel and others 2003; Madan and others 1999; Pories and others 1992). In addition to increasing the incidence of traumatic injury, alcohol intoxication also increases the severity of the injury as seen by greater injury severity scores, as well as an increased need for ICU, blood transfusions and surgery (Fabbri and others 2001). This contributes to worsened outcomes in the intoxicated trauma patient including increased mortality and acute medical complications such as respiratory failure and hemorrhagic shock (Fabbri and others 2001; Swearingen and others 2010; Tien and others 2006). Hemorrhagic shock accounts for over 35% of pre-hospital deaths and over 40% of deaths within the first 24 hours after traumatic injury (Wade and others 2006). After injury, alcohol-intoxication further disrupts traumatic injury-related hemodynamic instability (Shih and others 2003). Alcohol-intoxicated trauma patients are more hypotensive at the time of arrival to the emergency department compared to non-intoxicated individuals (Belillo and others 2011; Fabbri and others 2001; Shih and others 2003). Furthermore, intoxicated patients require greater 24 hr intravenous crystalloid fluid resuscitation volumes and significantly more blood products than non-intoxicated patients (Belillo and others 2011). A prospective cohort study examining the effects of alcohol intoxication on the severity and outcome from injury in 923 injured patients found that following the injury, alcohol-intoxicated patients had lower systolic blood pressure and greater morbidity and mortality (Shih and others 2033). Furthermore, results from clinical studies suggest that low mean arterial blood pressure (MABP) at the time of arrival into the emergency room is a predictor of poor patient outcome from traumatic injury and blood loss (Heckbert and others 1998; Heitsch and others 2001); therefore it is important to understand the mechanisms contributing to the greater hypotension potentially contributing to the increased morbidity and mortality seen in the alcohol-intoxicated trauma patient.

Studies from our laboratory utilizing a conscious, chronically catheterized rodent model of binge-like alcohol consumption preceding hemorrhagic shock have shown that AAI decreases basal MABP, exacerbates hypotension during hemorrhage, and attenuates blood pressure recovery during fluid resuscitation (Molina and others 2004). In response to a fixed-volume hemorrhage, alcohol-intoxicated animals are significantly more hypotensive throughout the hemorrhage and resuscitation period (Molina and others 2004). In response to a fixed-pressure (40 mmHg) hemorrhage, the hemodynamic deficits are apparent when examining the total volume of blood removed from each group to achieve the target pressure. A significantly lesser amount of blood is removed from the alcohol-intoxicated animals to achieve a fixed-pressure of 40 mmHg (Molina and others 2004). The greater hypotension and impaired hemodynamic stability during AAI was considered to be the potential result of several factors including an attenuated neuroendocrine response to hemorrhage, an impaired responsiveness to vasopressors released in response to blood loss, or a decreased blood volume at the time of hemorrhage.

Previous results demonstrated a significant attenuation of the counter-regulatory hormones and potent vasoconstrictors arginine vasopressin (AVP), epinephrine and norepinephrine during a fixed-pressure hemorrhage (Molina and others 2004). Additionally, when examining the neuroendocrine response to a fixed-volume hemorrhage, AAI was shown to result in an inappropriate neuroendocrine response for the exacerbated hypotension produced during the hemorrhage period (Molina and others 2004). Thus, in the AAI host, the impaired hemodynamic stability is associated with an impaired neuroendocrine

response to hemorrhage. Further investigations by demonstrated a critical role for central neuroendocrine activation of descending sympathetic pathways in the impaired responses to hemorrhage during AAI (Mathis and others 2009). Pharmacologic activation of the sympathetic nervous system (SNS) by intracerebroventricular (ICV) administration of neostigmine, an acetylcholine esterase inhibitor partially restored MABP and the catecholamine and AVP response to hemorrhage (Mathis and others 2009). These findings suggested that the integrity of the cells involved in releasing AVP in response to blood loss was preserved, yet the signaling pathways involved in triggering AVP release were disrupted by AAI. Thus, identification of the mechanisms underlying disregulated control of AVP release in AAI became an important focus of our investigations.

The possibility that decreased vascular responsiveness to prevailing pressor agents further contributed to the impaired hemodynamic recovery during AAI was considered. *In vitro* studies using isolated vessels (aortic and mesenteric arterioles) demonstrated that neither alcohol nor hemorrhage alone or in combination impair the pressor response to phenylephrine suggesting that the alcohol-induced impairments in blood pressure recovery following hemorrhage were not the result of an impaired vascular responsiveness to pressor agents (Molina and others 2009). Subsequently, whether AAI impairs the vascular responsiveness to pressor agents, particularly AVP, in an *in vivo* setting was determined.

In addition to an impaired neuroendocrine response to hemorrhage or decreased vascular responsiveness, the possibility that alcohol-treated animals had a significantly lower circulating blood volume at the time of hemorrhage was examined. Previous reports in the literature had suggested significant suppression of AVP release by AAI alone that could have contributed to a decreased blood volume during the period of alcohol infusion. Furthermore, we observed a significantly decreased basal MABP that could be the result of a decreased blood volume. Thus, studies were designed to investigate whether the greater hypotension during AAI could potentially be the result of a decrease in circulating blood volume.

Although improved resuscitation of trauma patients has dramatically reduced immediate mortality from hemorrhagic shock, long-term morbidity and mortality continue to be unacceptably high during the post-resuscitation period, particularly as a result of impaired host immune responses to subsequent challenges such as surgery or infection. Moreover, severity of trauma, hemorrhagic shock and injury is higher in intoxicated individuals than that of sober victims, resulting in higher mortality rates in this patient population. Necessary invasive procedures (surgery, anesthesia) and subsequent challenges (infection) that trauma victims are frequently subjected to are additional stresses to an already compromised inflammatory and neuro-endocrine milieu and further contribute to their morbidity and mortality. Thus, dissecting the dynamic imbalance produced by acute alcohol intoxication during trauma was the underlying aim of the studies performed under this project. Our results showed that acute alcohol intoxication at the time of hemorrhagic shock not only prevents adequate responses to fluid resuscitation but in addition impairs the ability of the host to overcome a secondary infection. The overall goals of the studies were to define the impact of acute ethanol intoxication on the immediate (neuro-endocrine, hemodynamic and pro-inflammatory) and delayed (susceptibility to infection) host responses to hemorrhagic shock and 2) To dissect the neuroimmune mechanisms involved in alcohol-induced dysregulation of host responses. We used a conscious, chronically instrumented rodent model of alcohol intoxication and trauma/hemorrhage to dissect the mechanisms responsible for impaired hemodynamic and host defense responses.

BODY:

Objective 1: To test the hypothesis that acute alcohol intoxication alters central activation of descending sympathetic outflow. The proposed studies will identify the mechanisms responsible for the impaired hemodynamic counter regulatory response to blood loss in the alcohol-intoxicated host. Specifically, to isolate central and peripheral regulatory mechanisms disrupted during alcohol intoxication.

Enhancing central nervous system cholinergic activity restores the neuroendocrine response to hemorrhagic shock in AAI

Several mechanisms could explain the alcohol-induced impairment of hemodynamic counter-regulation including a decrease in circulating blood volume prior to hemorrhage, an impaired vascular responsiveness to vasoactive substances, or a blunted neuroendocrine response to hemorrhage. Thus, both central and peripheral mechanisms could contribute to the observed effects. The impaired hemodynamic counter-regulation to hemorrhage in alcohol-intoxicated animals is associated with suppression of catecholamine (epinephrine and norepinephrine), and AVP responses to severe hemorrhage (MABP maintained at 40 mmHg for 60 minutes) (Molina 2004). Because catecholamines and AVP are the vasoactive hormones necessary for normal compensation to hemorrhage, blunting of their release during alcohol intoxication could be detrimental to hemodynamic stability following severe blood loss. Taken together, these observations led to the hypothesis that the decreased release of vasoactive hormones during hemorrhage is the principle mechanism by which alcohol acts to impair the recovery of MABP following blood loss. The initial studies determined whether enhancing central nervous system cholinergic activity can restore the neuroendocrine response to hemorrhagic shock and thereby improve hemodynamic counter-regulation and decrease morbidity and mortality in the alcohol-intoxicated host. Acute alcohol intoxication, produced by intragastric administration of alcohol, decreases baseline MABP, accentuates hypotension throughout hemorrhage, and blunts the pressor response to fluid resuscitation, regardless of the dose (1.75, 5, and 8 g/kg) and frequency of alcohol administration (single dose, three-day binge, and 15-h constant infusion, respectively) (Phelan, 2002; Mathis, 2006; Greiffenstein, 2007). This impaired hemodynamic counter-regulation following severe blood loss, where MABP is maintained at 40 mmHg for 60 minutes, is associated with attenuated plasma concentrations of catecholamines and AVP in alcohol-intoxicated animals (Molina, 2004). The inappropriate neuroendocrine response is hypothesized to be the principal mechanism underlying the hemodynamic instability observed in alcohol-treated animals following blood loss. In addition to impairing the neuroendocrine response, alcohol may decrease circulating blood volume, and/or reduce vascular reactivity to vasoactive hormones and neurotransmitters. Thus, alcohol could potentially exert its effects not only in peripheral tissues, but in the CNS as well.

The hypothesis that pharmacological interventions aimed at enhancing neuroendocrine activation could improve hemodynamic counter-regulation following hemorrhagic shock in alcohol-intoxicated animals was tested. In these studies, restoration of the neuroendocrine response to hemorrhage was accomplished by enhancing cholinergic activation in the CNS. Stimulation of CNS cholinergic neurons, an approach known to activate the SNS (Ulus, 1995), was predicted to improve hemodynamic compensation and recover MABP in alcohol-intoxicated, hypotensive rodents. Central choline administration was selected to dissect CNS from peripheral mechanisms involved in alcohol-induced impairment of the hemodynamic counter-regulatory responses to hemorrhage. The results from these studies demonstrated that ICV choline produced an immediate activation of the SNS as evidenced by the increase in MABP and the rise in plasma epinephrine, norepinephrine, and AVP within five minutes of administration in dextrose controls (Mathis et al, 2009, appended). Acute alcohol intoxication lowered basal MABP and prevented choline-induced neuroendocrine activation, but did not alter the pressor response of central choline administration. ICV choline produced few to no improvements in

hemodynamic and neuroendocrine counter-regulation in alcohol-intoxicated animals following hemorrhagic shock. The inability of ICV choline to reverse hemorrhage-induced hypotension and to produce a sustained increase in vasoactive hormone release throughout the duration of hemorrhage in alcohol-treated animals was attributed to transient enhancement of descending SNS outflow that was extinguished prior to the end of the hemorrhagic shock protocol. These studies demonstrated that ICV choline is capable of immediately activating the SNS, increasing MABP and sypathoadrenomedullary outflow, and increasing AVP release. However, due to its transient effect, ICV choline did not improve MABP at the end of hemorrhage and fluid resuscitation in dextrose controls or alcohol-treated rodents. This led to subsequent studies that investigated whether prolonging CNS cholinergic activity would improve hemodynamic counter-regulatory responses to hemorrhagic shock in the alcohol-intoxicated host. The premise was that increasing and sustaining CNS acetylcholine availability would lead to the desired response. Pilot studies tested the efficacy of ICV choline (150 µg) or ICV neostigmine (1 µg), an enzyme that inhibits degradation of acetylcholine, and ICV administration of a combination of the two drugs to prolong the MABP response. ICV neostigmine administration produced a greater increase in MABP in control rats than ICV choline administration alone, an increase which was sustained for longer periods (60 minutes versus 20 minutes). ICV neostigmine administration also increased plasma epinephrine and norepinephrine to a greater magnitude than ICV choline administration alone. These results led to the hypothesis that ICV neostigmine administration would produce a more sustained activation of SNS outflow and in turn would improve hemodynamic counter-regulation to hemorrhage in alcohol-treated animals.

Enhancing central nervous system cholinergic activity improves hemodynamic counter-regulation and decreases morbidity and mortality in the AAI host

Studies were designed to produce a sustained increase in SNS activation via ICV administration of the acetylcholinesterase inhibitor neostigmine. When injected directly into the CNS via the right lateral ventricle (i.e., ICV), neostigmine is confined to the brain, and functions by inhibiting the breakdown of acetylcholine into acetate and choline by acetylcholinesterase, therefore increasing acetylcholine availability (Wilkinson, 2004). Thus, any effects elicited by injection of this drug reflect a centrally-mediated response, which enables isolating these effects from potential systemic effects. The hypothesis for these studies was that ICV neostigmine administration would restore the neuroendocrine response to hemorrhage, and in turn, improve MABP recovery and outcome from hemorrhage during acute alcohol intoxication. To test this hypothesis, the effect of ICV neostigmine administration on SNS outflow was confirmed and the contribution of augmented catecholamine and arginine vasopressin responses to the pressor effects induced by CNS acetylcholinesterase inhibition was investigated. The effect of ICV neostigmine in reversing hemorrhage- or alcohol-induced hypotension was also investigated, before subjecting animals to a protocol that involved both alcohol and hemorrhage. Finally, in an effort to initiate translation of these basic science findings, the efficacy of systemic administration of physostigmine, an acetylcholinesterase inhibitor that readily penetrates the blood brain barrier, to produce similar activation of SNS responses as those observed with ICV neostigmine was demonstrated. The results from these studies demonstrated that CNS acetylcholinesterase inhibition is capable of improving hemodynamic compensation and outcome to hemorrhagic shock in alcohol-intoxicated rodents. The results showed that ICV neostigmine produced an immediate activation of SNS outflow reflected in increases in MABP, heart rate, and plasma catecholamines, AVP, and glucose. In addition, these studies provide strong evidence that the pressor response elicited by ICV neostigmine in both normotensive and alcohol-intoxicated, hypotensive animals is due to enhanced catecholaminergic and vasopressinergic activity. Overall, these results suggest that CNS acetylcholinesterase inhibition enhances SNS neurotransmission and improves hemodynamic compensatory responses to blood loss in alcohol-intoxicated rodents. Moreover, the results strongly

suggest that alcohol exerts its detrimental effects on hemodynamic counter-regulation to severe blood loss through CNS mechanisms.

Taken together, results from these studies and those reported in the literature indicate that ICV neostigmine enhances CNS cholinergic activity, activates the SNS, and improves catecholaminenergic and vasopressinergic responses to hemorrhagic shock in alcohol-intoxicated rodents. Restoring the neuroendocrine response through ICV neostigmine reverses hypotension during and following hemorrhagic shock in alcohol-intoxicated animals. These results suggest that during acute alcohol intoxication, the blunted neuroendocrine response plays a major role in the hemodynamic instability that follows hemorrhage. Studies investigating the efficacy of IV injection of physostigmine, which crosses the blood brain barrier, show a similar ability to enhance SNS activation in control animals. In order to translate these findings into a more clinically relevant scenario, it was important to examine whether similar responses could be elicited with the systemic administration of an acetylcholinesterase inhibitor. Moreover, the question arose as to whether the improvements in neuroendocrine responses and the restoration of MABP translated into improved tissue perfusion and thus decreased tissue injury. The objective of these studies was to determine whether systemic administration of an acetylcholinesterase inhibitor that crosses the blood brain barrier acts through similar mechanisms as ICV neostigmine to improve outcome from blood loss.

CNS acetylcholinesterase inhibition (via ICV neostigmine or IV physostigmine administration) produced an immediate activation of SNS in non-intoxicated animals. ICV neostigmine reversed hemorrhage- and alcohol-induced hypotension independently, and moreover, improved hemodynamic compensation following blood loss in alcohol-treated animals. The studies presented here demonstrated that IV physostigmine acts through CNS nicotinic receptor activation to increase MABP in normotensive alcohol-intoxicated animals as well. In addition, IV physostigmine reversed the hypotension associated with 40% blood loss and improved survival at one week despite the lack of fluid resuscitation in non-intoxicated animals. The reversal of hypotension is likely due to an augmented catecholamine response to blood loss. Finally, IV physostigmine enhanced the pressor response to fluid resuscitation in both dextrose controls and alcohol-treated animals during the recovery period of a more severe model of hemorrhage, where MABP was maintained at ~40 mmHg for 60 minutes. This enhanced pressor response to fluid resuscitation was mediated through CNS nicotinic receptors in dextrose controls, but not alcohol-intoxicated animals. The IV physostigmine-induced pressor response was also blunted by nicotinic antagonists at five minutes post injection in alcohol-treated animals.

The “life-saving” effects of intravenous administration of physostigmine following hemorrhage have long been identified in the literature (Savic, 1991) and were confirmed in this study. In fact, the ability of IV physostigmine to increase MABP was cited first in 1955 (Varagic, 1955; Dirnhuber, 1955); however, the mechanism of action was not established. Some suggest that the pressor effect of IV physostigmine is due to increases in residual blood volume in rabbits (Savic, 1991). However, the studies presented here suggested that the reversal of MABP following IV physostigmine injection in non-intoxicated animals was due to enhanced sympathoadrenomedullary activation, similar to other centrally-acting acetylcholinesterase inhibitors (neostigmine). The results from these studies demonstrate similar responses in the acute alcohol intoxicated host. IV physostigmine produced an immediate pressor effect in alcohol-treated animals at baseline, which was mediated through CNS nicotinic receptors. These findings confirm published reports that CNS muscarinic blockade does not inhibit increases in cervical preganglionic sympathetic nerve activity produced by IV physostigmine (Stamenovic, 1970). Studies have reported that the pressor effects of physostigmine are inhibited by scopolamine, a selective M_1 muscarinic receptor antagonist (Punnen, 1986). However, the results of these studies demonstrated that ICV atropine (nonselective muscarinic receptor antagonist) sulfate administration did not inhibit the pressor effects of IV physostigmine.

Augmented central nitric oxide production inhibits vasopressin release during hemorrhage in AAI

Having demonstrated that the impaired hemodynamic stability during AAI was primarily the result of a blunted neuroendocrine response, the mechanisms of this impaired response were next examined with focus on AVP. Previous studies had shown that central cholinergic stimulation by ICV administration of neostigmine, an acetylcholine esterase inhibitor, restores the AVP response to hemorrhage during AAI suggesting that AAI interferes with the central signaling mechanisms regulating AVP release during hemorrhagic shock (Mathis and others 2009). AVP release is regulated by a number of mediators including stimulation by ANG II and inhibition by NO. While ANG II has been shown to stimulate AVP release, NO exerts tonic inhibition on the PVN suppressing AVP release (Kadekaro and others 1998). Alcohol intoxication (4.5g/kg intra-peritoneal) results in a significant increase in NO in the plasma, anterior pituitary, and PVN of the hypothalamus (Seo and others 2003). Furthermore, AAI also increases NOS activity in the anterior pituitary and PVN (Seo and others 2003). Thus, under normovolumic and iso-osmotic conditions, AVP release is inhibited by NO; while during hemorrhage, AVP release is stimulated by ANG II. The balance between ANG II stimulation and NO inhibition is essential for producing an appropriate AVP response. Because NO inhibits AVP release, and NO production in the PVN has been shown to be increased in AAI, we hypothesized that increased inhibitory nitrergic tone contributes to the alcohol-induced attenuation of the AVP response to hemorrhage and would impair the AVP response to hyper-osmolality**.** Studies were designed to examine the nitrergic regulatory mechanisms modulating AVP release in response to hemorrhage and hyper-osmolality. Furthermore, we sought to demonstrate that removing the inhibition of NO would restore the AVP response to hemorrhage and improve MABP in AAI.

Previously, we had demonstrated that AAI markedly attenuated the AVP response to hemorrhagic shock (Molina and others 2004). In these studies we observed a significant increase in NO content, a known inhibitor of AVP release, in the hypothalamic PVN and SON at the completion of the alcohol infusion. NO content remained significantly elevated in the PVN of alcohol-intoxicated animals at the completion of hemorrhage. In contrast, AAI did not impair the AVP response to an osmotic challenge. This was accompanied by a significant decrease in NO content in the PVN of alcohol-intoxicated animals. To confirm the role of NO in the alcohol-induced inhibition of AVP release during hemorrhage, the NOS inhibitor, L-NAME, was administered centrally prior to hemorrhage. The NOS inhibitor, L-NAME, was administered ICV 15 minutes prior to hemorrhage at a dose of 250 µg/ 5 µl. L-NAME was selected because it is a non-selective NOS inhibitor (inhibiting nNOS, eNOS and iNOS) allowing for complete NOS inhibition. The dose was selected based on observations by Kadekero et al. demonstrating significant NOS inhibition in the PVN immediately following ICV administration of this dose of L-NAME (Kadekaro and others 1998). Additional studies have demonstrated an onset of NOS inhibition within15 minutes after administration and effects that last up to 6 hours in several brain structures following ICV administration L-NAME; therefore, we chose to wait 15 minutes following ICV administration of L-NAME prior to initiating the fixed-pressure hemorrhage to allow for maximal NOS inhibition (Salter and others 1995). To confirm NOS inhibition by L-NAME in our model, NO content and NOS activity were measured in the hypothalamic PVN and SON at the completion of hemorrhage. In the present study, we observed a 41% decrease in NO content and a 35% decrease in NOS activity in the PVN of dextrose/LNAME-treated animals compared to dextrose/vehicle-treated animals at the end of hemorrhage (75 minutes after L-NAME administration). In alcohol-treated animals, ICV administration of L-NAME resulted in a 44% decrease in NO content and a 61% decrease in NOS activity in the PVN compared to alcohol/vehicle-treated animals at the completion of the hemorrhage period. NO content in the SON, however, was not altered by ICV L-NAME administration, which could potentially be attributed to limited access of the drug to this region. The effects of L-NAME on hemodynamic stability in this model are evident when examining the total amount of blood removed during the 60 minute hemorrhage

period to achieve a fixed-pressure (~40 mmHg) hemorrhage. All animals were brought to a similar degree of hypotension throughout the hemorrhage period. However, alcohol-treated animals required a significantly less amount of blood removed during the hemorrhage period to achieve a fixed-pressure of ~40-50 mmHg as compared to dextrose-treated animals. Administration of L-NAME increased the amount of blood removed to achieve the target pressure suggesting that the animals have an enhanced compensatory response to the hemorrhage as confirmed by restoration of the circulating levels of AVP following hemorrhage.

In contrast to the inhibitory effects of NO, AVP release is stimulated by ANG II. In this study, ANG II was measured at baseline, 5 minutes, 20 minutes, and 60 minutes during the hemorrhage. There was a significant delay in the hemorrhage-induced increase in ANG II in the alcohol-treated animals at the 20 minute time point. Studies have demonstrated that peripheral ANG II can enhance AVP release resulting in significantly elevated circulating AVP levels (Lee and others, 1995). Thus, the elevated circulating ANG II levels following central L-NAME administration may have contributed to the restoration of circulating AVP levels during AAI. In addition to the well known effects of ANG II of vasoconstriction, ANG II exerts stimulatory effects in the PVN through inhibition of pre-synaptic release of NO and GABA (Li and others, 1983). NO potentiates GABA release and the resulting inhibition of sympathetic outflow (Zhang and others, 1998). Thus, the increased circulating ANG II levels could also have contributed to enhanced activation of sympathetic pathways in the PVN as well as other brain areas through AT_1 receptor-mediated stimulation or by potentially decreasing NO-mediated and GABA-mediated inhibition of neuronal activity of the PVN. Nevertheless, the results from this study clearly demonstrate that central NOS inhibition restores the AVP response to hemorrhage during AAI. Taken together, the results from these studies strongly suggested that the alcohol-induced impairment of the AVP response to hemorrhage is the result of augmented central NO inhibition. However, the mechanisms underlying the alcohol-induced modulation of NO production in the PVN remained unknown.

<u>Objective 2:</u> Examine the impact of alcohol intoxication on vascular responsiveness to pressor agent administration.

Alcohol does not Modulate the Augmented Acetylcholine-induced Vasodilatory Response in Hemorrhaged Rodents

Using isolated vascular rings, we examined the impact of a binge model of acute ethanol intoxication on vascular reactivity to pressor agents following hemorrhagic shock and fluid resuscitation. These studies examined whether the greater hypotension seen in AAI could be the result of a decreased vascular responsiveness to pressor agents (Molina and others 2009). In this study, superior mesenteric artery and aortic rings were isolated either at the completion of the alcohol infusion, at the completion of the hemorrhage, or at completion of fluid resuscitation with lactated Ringer's. Results from those studies showed that increasing doses of the vasoconstrictor phenylephrine [1×10^{-9} M] to [1×10^{-5} M] produced a dose dependent increase in tension in all vessels regardless of the time at which they had been excised. Our results showed that second messenger signaling in response to changes in membrane potential is intact, as reflected by preserved KCl-medicated contraction in vessels isolated from both control and alcohol-intoxicated animals. Moreover, receptor mediated contraction, tested with the alpha-adrenergic agonist phenylephrine also was shown to be unaffected by alcohol. The results suggest that this is not a principal mechanism associated with the impaired hemodynamic instability in alcohol-intoxicated hemorrhaged animals and suggest a central role for the attenuated neuroendocrine responses observed in previous studies. No effects of alcohol, hemorrhage, or the combination of AAI and hemorrhage were noted suggesting that impaired vascular responsiveness to pressor agents such as adrenergic agonists was not the underlying mechanism for greater hypotension or blunted recovery of blood pressure in AAI animals. Although responsiveness to AVP was not examined *in vitro* due to

technical difficulties, its effectiveness in producing a pressor response in AAI hemorrhaged animals was examined in vivo. Taken together, the results from previous studies had provided strong support for a central role of blunted counteregulatory responses to hemorrhage as the underlying mechanism for impaired hemodynamic recovery from hemorrhage in AAI. To eliminate the possibility that additional mechanisms played an important role in the impaired hemodynamic recovery to hemorrhage in AAI animals, two additional studies were designed.

Impaired vascular reactivity and decreased blood volume do not contribute to impaired hemodynamic recovery from hemorrhage in AAI

Our results demonstrated that circulating blood volume and vascular response to *in vivo* pressor administration are not affected by AAI, and thus do not contribute to the impaired hemodynamic responses to hemorrhage. AAI resulted in a marked early increase in urine output and decreased urine osmolality as compared to dextrose-infused and no infusion controls; however, at the completion of the infusion at the time when blood volume was measured, there were no differences in these parameters. This was associated with a significant, yet transient increase in plasma osmolality 2 hours into the alcohol infusion, which had normalized at the completion of the alcohol infusion; the time at which we normally initiated the hemorrhage protocol. The results from these studies demonstrated an early alcohol-induced diuresis that was not associated with suppression of circulating AVP levels. This would be in contrast to previous reports in the literature indicating that alcohol ingestion is followed immediately by suppression of AVP. The alcohol-induced diuresis at the 1 hour and 2 hours time point could have potentially contributed to the increased plasma osmolality, which we would predict stimulated vasopressin release, renal water reabsorption, and preserved circulating blood volume.

Taken together, these results along with our previous findings suggest that the greater hypotension in AAI is not due to a decreased circulating blood volume or a decreased vascular responsiveness to counter-regulatory hormones leaving a blunted neuroendocrine response as the most likely candidate for the impaired counter-regulatory response to hemorrhagic shock in AAI. Furthermore, these results provided evidence that AAI did not alter the vascular responsiveness to pressor agents, as both *in vivo* and *in vitro* studies demonstrated intact pressor responses during hemorrhage and fluid resuscitation. Moreover, these results suggested that the rise in circulating AVP levels significantly contributes to restoration of MABP following hemorrhage and that this is not impaired in AAI.

<u>Objective 3:</u> To test the hypothesis that the alterations in hemodynamics produced by acute alcohol intoxication during trauma-hemorrhage result in inadequate tissue perfusion during the resuscitation period leading to enhanced susceptibility to tissue injury.

Alterations in hemodynamics produced by AAI during trauma-hemorrhage result in inadequate tissue perfusion during the resuscitation period leading to enhanced susceptibility to tissue injury

Organ blood flow was determined by the reference sample technique utilizing fluorescent microspheres at 2 hours post-resuscitation. In addition, tissue and blood samples were obtained to determine markers of tissue injury. Combined, this approach allowed for the demonstration of the impact of AAI on organ blood flow and tissue injury and dysfunction. AAI alone did not alter organ blood flow. In contrast, hemorrhagic shock in AAI animals resulted in significant reduction of blood flow to the liver (72% reduction vs sham; $p<0.05$), kidney (63% reduction vs sham; p=NS), small intestine (65% reduction vs sham; $p<0.05$), and large intestine (67% reduction vs sham; $p<0.05$). Circulating levels of alanine aminotransferase (ALT) were not altered by AAI or by hemorrhagic shock alone. In contrast, ALT was significantly increased in AAI-hemorrhaged animals (~4-fold above time-matched AAI-sham). In addition, creatinine was significantly elevated in AAI-hemorrhaged animals. In additional studies, we determined whether the alterations in tissue blood flow were associated with disrupted $PGE_2$and NO control mechanisms in the gut and whether these, in conjunction with excessive generation of reactive oxygen

species contributes to loss of barrier function. Our results showed that AAI alone did not alter gut PGE_2 content in time-matched shams 24 hours post-HS. HEMORRHAGIC SHOCKled to a significant suppression of gut PGE_2 content in both dextrose- (42 % decrease vs time-matched sham) and AAI-treated animals (45% decrease vs time-matched sham and 51% decrease vs dextrose sham). PGE_2 levels had a significant negative correlation with FD-4 translocation, the selected marker of intestinal permeability (r= -0.56; p=0.019). Hemorrhagic shock and resuscitation with LR resulted in a robust, but variable and non-statistically significant increase in protein carbonylation (reflecting oxidative stress). Hemorrhagic shock during AAI produced a significant ~8.6-fold increase in protein carbonyl content. Hemorrhagic shock in dextrose-treated animals resulted in increased ~4000 kD FITC-labeled dextran translocation following resuscitation and this was markedly enhanced in AAI-hemorrhaged (~4.9-fold increase vs dextrose-sham). Taken together, these findings indicated that the impaired tissue blood flow was associated with altered tissue function.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated that acute alcohol intoxication alters sympathetic outflow
- Demonstrated that alcohol intoxication increases mortality from trauma/hemorrhage
- Established the feasibility of using ICV choline to activate sympathetic outflow and increase blood pressure
- Determined that alcohol intoxication decreased blood pressure and blunts the choline-induced rise in norepinephrine
- Demonstrated that ICV choline does not improve hemodynamic response to hemorrhage
- Observed that the activation of the SNS by ICV choline does not appear to be sustained throughout the duration of the hemorrhage
- Determined that alcohol intoxication during hemorrhage blunts host defense response to a second-hit challenge in PBMCs even after a 24 h recovery
- Demonstrated that alcohol intoxication in vivo does not alter vascular reactivity to presser agents
- Demonstrated that neostigmine reverses hypotension produced by blood loss in control and alcohol-intoxicated animals
- Demonstrated that the effects of neostigmine are mediated in part by arginine vasopressin respetor activation
- Demonstrated that survival from hemorrhage in alcohol-intoxicated animals can be significantly improved by restoring blood pressure closer to basal values
- Demonstrated that alcohol decreases the peak tension achieved with vasopressors and accentuates the vasodilation observed with acetylcholine
- Demonstrated that in vivo, the responsiveness to arginine vasopressin is preserved in alcohol-intoxicated hemorrhaged animals.
- Demonstrated that neostigmine produces significant increases in catecholamines in response to blood loss during AAI
- Demonstrated that improving blood pressure recovery following hemorrhagic shock leads to an improved pro-inflammatory cytokine response to a subsequent systemic challenge
- Demonstrated that central cholinergic activation plays a role in the dysregulation of the inflammatory response following hemorrhage in AAI
- Demonstrated that enhanced nitric oxide tone in the hypothalamus of the AAI host suppresses AVP release during hemorrhage
- Demonstrated that AAI disrupts tissue perfusion and blood flow distribution following hemorrhage and resuscitation
- Established that the altered tissue perfusion is associated with enhanced tissue injury and gut permeability
- Showed that AAI does not alter the ability of the host to increase AVP release following increased osmolarity

REPORTABLE OUTCOMES:

Publications:

1. Mathis KW, Zambell K., Olubadewo JO, Molina, PE. Altered hemodynamic counter-regulation to hemorrhage by acute moderate alcohol intoxication. Shock 26(1):55-61, 2006.
2. Greiffenstein P, Mathis KW, Vande Stouwe C, Molina PE. Alcohol binge prior to trauma/hemorrhage impairs integrity of host defense mechanisms during recovery. Alcohol Clin Exp Res. 31(4):704-15, 2007.
3. Greiffenstein P, Mathis KW, Vande Stouwe C, Molina PE. Alcohol binge prior to trauma/hemorrhage impairs integrity of host defense mechanisms during recovery. Alcohol Clin Exp Res. 31(4):704-15, 2007.
4. Greiffenstein P, Molina PE. Alcohol-Induced Alterations of Host Defense Following Traumatic Injury. J. Trauma 64(1):230-40, 2008.
5. Bird MD, Choudhry MA, Molina PE, Kovacs EJ. Alcohol and trauma: a summary of the Satellite Symposium at the 30th Annual Meeting of the Shock Society. Alcohol. 43(3):247-52, 2009. PubMed PMID: 19393863.
6. Molina MF, Whitaker A, Molina PE, McDonough KH. Alcohol does not Modulate the Augmented Acetylcholine-induced Vasodilatory Response in Hemorrhaged Rodents. Shock. 32(6):601-7, 2009. PubMed PMID: 19197228.
7. Mathis K, Molina PE. Transient central cholinergic activation enhances sympathetic nervous system activity but does not improve hemorrhage-induced hypotension in alcohol-intoxicated rodents. Shock. 32(4):410-5, 2009. PubMed PMID: 19197225.
8. Mathis KW, Molina PE. Central acetylcholinesterase inhibition improves hemodynamic counter-regulation to severe blood loss in alcohol-intoxicated rats. Am J Physiol Regul Integr Comp Physiol. 297(2):R437-45, 2009 PubMed PMID: 19515985.
9. Mathis KW, Sulzer J, Molina PE. Systemic administration of a centrally-acting acetylcholinesterase inhibitor improves outcome from hemorrhagic shock during acute alcohol intoxication. Shock 34(2):162-8, 2010. PubMed PMID: 20023599.
10. Whitaker AM, Sulzer J, Walker E, Mathis K, Molina PE. Sympathetic Modulation of the Host Defense Response to Infectious Challenge during Recovery from Hemorrhage. Neuroimmunomodulation 17:349-358, 2010. PMID: 20516716.
11. Sulzer J, Molina PE. Delayed resuscitation with physostigmine increases end organ damage in alcohol intoxicated rats. Shock. 35(1):74-9, 2011. PMID: 20577152
12. Souza-Smith FM, Kurtz KM, Molina PE, Breslin JE. Adaptation of Mesenteric Collecting Lymphatic Pump Function Following Acute Alcohol Intoxication. Microcirculation 17(7):514-24, 2010. PMID: 21040117
13. Whitaker AM, Sulzer JK, Molina PE. Augmented nitric oxide production in the paraventricular nucleus inhibits vasopressin release during hemorrhage in acute alcohol intoxicated rodents. 301(5):R1529-39, 2011. Epub 2011 Aug 17. PMID: 21849630
14. Sulzer JK, Whitaker AM, Molina PE. Hypertonic saline resuscitation enhances blood pressure recovery and decreases organ injury following hemorrhage in acute alcohol intoxicated rodents. Submitted J. Trauma Dec 2011

Presentations:

1. Acetylcholinesterase inhibitor improves survival from hemorrhage in rodents. FASEB, New Orleans, LA, April 2009.

2. Alcohol-induced hemodynamic instability during hemorrhagic shock is reversed by acetylcholinesterase inhibitor. *Alcoholism: Clinical and Experimental Research.* 2008;32(6S):30A. Washington DC, July 2008.
3. Central neostigmine administration reverses alcohol- and hemorrhage-induced hypotension. (1227.9) FASEB, San Diego, CA, April 2008.
4. Effective mechanisms in restoring blood pressure following hemorrhage. (1227.16) FASEB, San Diego, CA, April 2008.
5. Central choline-mediated sympathetic outflow does not attenuate hypotensive response to blood loss in acute alcohol-intoxicated rodents. Research Society on Alcoholism. *RsoA*. Chicago, IL, July 2007.
6. Short-term central activation of descending sympathetic outflow does not restore alcohol-induced hemodynamic instability during hemorrhagic shock. FASEB, Washington, DC, April 2007.
7. Acute alcohol intoxication does not modulate cardiovascular and neuroendocrine response to immobilization stress. *RSoA* Baltimore, MD, June 2006.
8. Alcohol binge suppresses early lung TNF expression following trauma and hemorrhagic shock. FASEB, San Francisco, CA, April 2006.
9. Short-term moderate alcohol intoxication impairs hemodynamic and immune response to hemorrhagic shock. FASEB, San Diego, CA, April 2005.
10. Alcohol binge suppresses early lung TNF expression following trauma and hemorrhagic shock. EB, San Francisco, April 2006.
11. Impact of acute moderate alcohol intoxication on outcome from hemorrhage. Experimental Biology, San Francisco, April 2006.
12. Impact of alcohol intoxication on outcome from hemorrhagic shock. VII Congreso Internacional en Adicciones y 5 Curso de Actualización en Adicciones: de la Ciencia Básica a la Clínica. Saturday, April 29th, 2006. Guadalajara, Mexico.
13. Contribution of soft tissue trauma to pro-inflammatory responses to shock and trauma. Shock Society Meeting, Denver Colorado. June, 2006.
14. Acute alcohol intoxication does not modulate cardiovascular and neuroendocrine response to immobilization stress. Research Society on Alcoholism, Baltimore, July 2006.
15. Impact of Alcohol Intoxication on Outcome from Trauma/Hemorrhage. Department of Physiology, Tulane University, February 18, 2008.
16. Alcohol interaction with host defense in traumatic injury. Alcohol, Leukocytes, and host defense Satellite Symposia. Denver, CO Nov 9, 2008.
17. Targeted pharmacotherapies to improve outcomes from shock in the alcohol-intoxicated host. Department of Defense Military Health Research Forum Kansas City, Missouri, September, 2009
18. Alcohol binge suppresses early lung TNF expression following trauma and hemorrhagic shock. FASEB, San Francisco, April 2006.
19. Alcohol intoxication impairs counteregulatory responses during hemorrhagic shock. Louisiana Academy of Sciences, Southern University, Baton Rouge, LA (March 2007)
20. Noradrenergic modulation of systemic response to infection. Society on Neuroimmune Pharmacology. Salt Lake City, April 2007.
21. Short-term central activation of descending sympathetic outflow does not restore alcohol-induced hemodynamic instability during hemorrhagic shock. FASEB, Washington, DC, April 2007.
22. Acute alcohol intoxication impairs counteregulatory responses during hemorrhagic shock. Graduate Research Day, LSU Health Sciences Center, New Orleans, LA, April 2007.

23. Consequences of alcohol intoxication during trauma/hemorrhage. Shock Society Meeting Satellite, Baltimore, MD, June 2007.
24. Transient sympathetic nervous system activation does not alter early tissue inflammatory responses to hemorrhagic shock. Shock Society Meeting, Baltimore, MD, June 2007.
25. Central choline-mediated sympathetic outflow does not attenuate the hypotensive response to blood loss in acute alcohol-intoxicated rodents. Alcohol and Trauma 2007, Shock Society Satellite Meeting, Baltimore, MD, June 2007.
26. Central choline-mediated sympathetic outflow does not attenuate hypotensive response to blood loss in acute alcohol-intoxicated rodents. Research Society on Alcoholism. Chicago, IL, July 2007.
27. Central manipulation of the sympathetic nervous system during hemorrhagic shock in the alcohol-intoxicated host. NIAAA Trainee Workshop, Indianapolis, IN, September 2007.
28. Alcohol intoxication and traumatic injury; Hemodynamic, metabolic and immune dysregulation. European Society for Biomedical Research in Alcoholism, Berlin, Germany, September 2007.
29. Impact of alcohol intoxication on outcome from trauma/hemorrhage. Tulane University, New Orleans, February 2008.
30. Alcohol Intoxication, Impact on Hemorrhagic Shock. 11th Capital City Conference of the German Society of Anesthesiology and Intensive Care Medicine, September, 2009.
31. Cardiovascular, metabolic and immune consequences of alcohol intoxication in the trauma victim. Pharmacology Research Seminar, LSUHSC March 2010.

CONCLUSION:

Acute alcohol intoxication (AAI) impairs the hemodynamic response to hemorrhagic shock (HS) and fluid resuscitation (FR) with lactated Ringer's (LR), attenuates the HS-induced rise in plasma arginine vasopressin (AVP), and increases organ injury following HS. Studies demonstrated a critical role for central neuroendocrine activation of descending sympathetic pathways in the impaired responses to HEMORRHAGIC SHOCK during AAI. In the intoxicated host, central administration of the acetylcholinesterase inhibitor neostigmine, which increases sympathetic outflow, restores the neuroendocrine and subsequently the hemodynamic response to a fixed pressure hemorrhage in which 50% of the estimated blood volume was removed. These studies were expanded to investigate the therapeutic potential of this approach by utilizing the peripherally administered acetylcholinesterase inhibitor physostigmine. As was observed with direct central acetylcholinesterase inhibitor administration, intravenous administration of physostigmine at the time of fluid resuscitation improves the neuroendocrine response and blood ressure recovery in intoxicated animals following HS. These effects were demonstrated to be mediated by central nicotinic receptors. Combined, these studies provided support for the overarching hypothesis that AAI disrupts central pathways involved in the neuroendocrine and sympathetic responses to blood loss. It was predicted that the improvement in hemodynamic recovery from hemorrhage in the alcohol-intoxicated host would also ameliorate the long term metabolic and inflammatory consequences to hemorrhage leading to reduced morbidity and mortality. Indeed, physostigmine at the time of fluid resuscitation was able to attenuate organ damage in alcohol-hemorrhage animals, as measured by circulating levels of alanine amino transferase (ALT) and blood urea nitrogen (BUN), markers of hepatic and renal damage and dysfunction respectively.

To examine if physostigmine could still be utilized as a 'rescue' in the intoxicated host following delayed resuscitation from hemorrhage, we subjected intoxicated animals to a one hour delay following hemorrhagic shock (HS) prior to fluid resuscitation with or without physostigmine. All animals demonstrated some degree of autoresuscitation during the delay, but remained hypotensive. At the completion of the delay period, lactated Ringers (LR) resuscitation failed to significantly increase mean arterial blood pressure above end-delay values. In contrast, systemic acetylcholinesterase inhibitor administration was still effective in enhancing mean arterial blood pressure recovery even after a one hour delay. However, while the improved pressor response to fluid resuscitation achieved with acetylcholinesterase inhibitor administration was associated with attenuation of organ injury when administered immediately following hemorrhage, delayed administration, following 120 minutes of hypotension, resulted in significantly greater organ injury. A possible explanation for the accentuated tissue injury seen in these studies is that the combination of the potent vasopressor response of acetylcholinesterase inhibitors with low blood flow states present in many organ beds during the hypotensive and resuscitation periods can lead to a critical state of tissue hypoperfusion and subsequent tissue damage. The kidney and liver are particularly vulnerable to increased damage with pressor use during shock periods. Indeed, those studies demonstrated that alanine aminotransferase (ALT) and aspartate aminotransferase (AST), markers of hepatic injury and dysfunction, as well as blood urea nitrogen (BUN) and creatinine, makers of renal dysfunction, were elevated following delayed resuscitation with acetylcholinesterase inhibitor in intoxicated animals. In addition to circulating makers of damage and dysfunction, levels of liver protein carbonyls, a stable maker of oxidative injury, were found to be elevated following delayed resuscitation with physostigmine during AAI. This suggested not only a role for impaired perfusion, but enhanced tissue damage resulting from excess generation of reactive oxygen species.

In addition, we demonstrated that AAI prior to hemorrhage disrupts the balance of protective and injurious agents in the gut contributing to an accentuated loss of intestinal barrier function during recovery from HS. Hemorrhagic shock decreased levels of PGE_2, which is critical in the maintenance of

barrier function, and the combination of AAI and hemorrhagic shock significantly increased NO content, which directly impairs intestinal epithelial cell barrier function, in the gut after a 24 recovery period. These alterations in tissue function were associated with marked impairment in tissue blood flow in AAI/hemorrhaged animals. Taken together, these findings provide substantial evidence for a role of central modulation of the neuroendocrine system in the response to hemorrhage during AAI. They identify NO inhibitory tone as a principal mechanism for AAI-mediated suppression of AVP in response to hemorrhagic shock. Furthermore, they strongly support an important vasopressor contribution of AVP to recovery from hemorrhage that is of critical relevance in the AAI host. Of interest, is the fact that AAI does not produce a non-specific and generalized effect on AVP release, but that it is exclusive to hemorrhagic shock.

In summary, our observations support a neuroendocrine mechanism for alcohol-induced derangement in hemodynamic recovery from hemorrhagic shock. We show that attenuated release of important counteregulatory hormones, particularly AVP is mediated by disrupted central signaling mechanisms including nitric oxide and sympathetic nervous system. However, the vasopressor response to exogenously administered agents does not appear to be affected by AAI. Of translational relevance is the unique involvement of nitric oxide as a suppressive agent in hemorrhage induced AVP release, a mechanism that can be overridden by hypertonic saline resuscitation.

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